IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	Examiner: D. Sullivan
CHOO et al.	Group Art Unit: 1636
Serial No.: 09/996,484	Confirmation No.: 2713
Filing Date: November 28, 2001	Customer No.: 20855
Title: MOLECULAR SWITCHES	

BRIEF ON APPEAL UNDER 37 C.F.R. § 41.37

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Commissioner for Patents
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Sir:

This Appeal Brief is filed in response to the Final Office Action mailed on October 30, 2006 and Advisory Action mailed on February 14, 2007. A Notice of Appeal was mailed on January 30, 2007 and received in the USPTO on February 1, 2007, making an Appeal Brief initially due on or before April 1, 2007. Appellants hereby request a one-month extension of time and attach the appropriate fee, thereby extending the deadline to May 1, 2007. Accordingly, this Brief is timely filed.

REAL PARTY IN INTEREST

Gendaq Ltd. is the assignee of the instant application, as recorded on August 22, 2005 in the USPTO at Reel 016655, Frame 0867. See, also, Certificate Under 37 C.F.R. § 3.73(b) filed on April 1, 2002. Gendaq, Ltd. is a wholly owned subsidiary of Sangamo BioSciences, Inc. Therefore, the real party in interest is Sangamo BioSciencies, Inc.

RELATED APPEALS AND INTERFERENCES

In *Ex parte Cox et al.* (Appeal No. 2006-1270; USSN 10/222,614), construction of the claim terms "first protein" and "second protein" was addressed by the Board. *See* Decision mailed September 27, 2006 at pages 7-8. Inasmuch as construction of these terms is relevant to the outstanding rejection under 35 U.S.C. § 103(a) in the present case, a copy of that decision is attached.

STATUS OF CLAIMS

Claims 1, 2, 4, 5, 7, 8, 10, 11, 13-15, 21-26, 31, 34, 35 and 38-48 are pending and claims 1, 2, 4, 5, 7, 8, 10, 11, 13-15, 21-26, 31, 35 and 38-47 have been withdrawn from consideration. Accordingly, claims 34 and 48 are under appeal.

STATUS OF AMENDMENTS

No amendments have been made subsequent to the mailing of the Final Office Action on October 30, 2006.

SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 34 is drawn to a complex (page 10, lines 16-19) comprising (a) a heterodimer comprising first and second polypeptides (page 2, lines 8-11) and (b) a ligand (page 10, lines 18-19). The first and second polypeptides bind to DNA, and, in addition, the first or second polypeptide comprises an engineered Cys2-His2 zinc finger binding domain (page 23, line 4 through page 31, line 31).

Independent claim 48 is drawn to a switching system comprising a protein switch (page 5, lines 14-15) comprising: (i) a first component comprising a first polypeptide and (ii) a second component comprising a second polypeptide (page 5, lines 15-16), in which the first polypeptide binds to the second polypeptide in a manner modulatable by a ligand (page 5, line 14), and (iii) a third component comprising the ligand, wherein the first and second polypeptides bind to DNA (page 5, lines 18-20), and further wherein the first or second polypeptide comprises an engineered Cys2-His2 zinc finger binding domain (page 23, line 4 through page 31, line 31).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claim 34 is anticipated under 35 U.S.C. § 102(b) by WO 95/19431 (hereinafter "Barbas").

B. Whether claim 48 would have been obvious under 35 U.S.C. § 103(a) based on WO 93/23431 (hereinafter "Vegeto"), as evidenced by McEwan *et al.* and Bledsoe *et al.* in light of Liu *et al* (1997) *Proc. Nat'l. Acad. Sci. USA* 94:5525-5530 (hereinafter "Liu")

ARGUMENTS

A. Claim 34 is not anticipated by the cited reference

Claim 34 stands finally rejected under 35 U.S.C. § 102(b) as allegedly anticipated by WO 95/19431 (hereinafter "Barbas").[1] It was alleged that the broadest reasonable interpretation of the claim term "ligand" embraces "anything that binds to either one of the first and second polypeptide," including a zinc ion:

> As discussed in the Final Office Action, "[T]he specification states at p. 49, ll. 1-2, 'A ligand according to the invention is typically any molecule capable of binding to any of the other components of a switching system.' Thus, the amended claim [which no long requires that the polypeptides bind in a manner modulatable by a ligand] now embraces any complex comprising a heterodimer comprising a first and second DNA-binding polypeptide and anything that binds to either one of the first and second

[1] Final Office Action, October 30, 2006, pages 7-8

> polypeptide..." (30 October 2006 Office Action, page 7.) As further stated at page 8 of the Final Office Action, "On page 1, ¶4, Barbas et al. teaches that zinc finger domains are folded around a zinc ion. As the zinc ion is bound to the first and second protein moieties, zinc is a ligand according the broadest reasonable interpretation of the claim limitation." Thus, the heterodimer of Barbas et al. does, in fact, comprise a ligand and anticipates every element of the claimed invention. (Advisory Action, Feb. 14, 2007, page 2.)

Appellants submit that claim 34 is not anticipated by Barbas because Barbas's Zif(C7)$_6$-Jun/Zif-268-Fos complex is not a complex including a heterodimer component and a ligand component, as set forth in claim 34.

Anticipation is a rigorous standard – every limitation of the claim at issue must appear identically in a single reference for a rejection under 35 U.S.C. § 102 to stand. *In re Bond,* 910 F.2d 831, 832, 15 USPQ2d 1566, 1567 (Fed. Cir. 1990). Moreover, the claim must first have been properly construed to define the scope and meaning of each limitation. See, *e.g., In re Paulsen*, 30 F.3d 1475, 1479, 31 USPQ2d 1671, 1674 (Fed. Cir. 1994) ("To properly compare [an allegedly anticipatory reference] with the claims at issue, we must construe the term 'computer' to ascertain its scope and meaning.").

1. Claim construction: "ligand"

In construing claim 34, it must be kept in mind that "as an initial matter, the PTO applies to the verbiage of the proposed claims the broadest reasonable meaning of the words in their ordinary usage as they would be understood by one of ordinary skill in the art, taking into account whatever enlightenment by way of definitions or otherwise that may be afforded by the written description contained in the applicant's specification." *In re Morris*, 127 F.3d 1048, 1054, 44 USPQ2d 1023, 1027 (Fed. Cir. 1997). The teachings of the specification as a whole must be taken into account, particularly what is taught about the claim terms in the particularly claimed context.[2]

In the present case, the specification provides considerable insight into the meaning of the term "ligand" in the context of a complex comprising a heterodimeric

[2] *See* also *Phillips v. AWH*, 75 USPQ2d 1321 (Fed. Cir. 2005)

protein and the ligand. In particular, the specification makes clear, with reference to various scientific papers, that, in the claimed protein switches, protein-binding ligands are molecules made up of two or more atoms (page 49, lines 16-28):

> As applied to a protein switch, a ligand is any molecule capable of binding to the polypeptide binding molecule (including a polypeptide binding protein), or another protein. Protein binding ligands are known in the art, and include, for example, immunoglobulins, antibodies, ATP, cAMP, GABA, Fas ligand, CIDs (chemical inducers of dimerization), an FK506 and FK1012 (as described in Spencer et al. 1993, Science 262 1019), peptide hormone molecules, retinoic acid, acridine derivatives and other anticancer drugs as described in Finlay and Baguley (2000), Cancer Chemother Pharmacol 45. 417. etc.
>
> Ligand mediated protein-protein association is described for example in Lin et al (1998), Blood 91, 890-897, Spencer et al (1993), Science 262, 1019, Keenan et al. (1998) Bioorganic and Medicinal Chemistry 6, 1309-1335 and Fan et al. (1999), Human Gene Therapy 10, 2273-2285.

In addition, the specification also makes clear that, in the context of a complex comprising a heterodimer and a ligand (*i.e.*, a protein switch), the ligand component of the complex is separate from the polypeptides and this separate ligand component modulates binding of the two proteins (page 10, lines 16-19 and lines 30-33):

> The term "complex" is used to describe an association between a DNA and one or more molecules as defined herein, or between a polypeptide molecule and one or more molecules. In the case of a polypeptide, these molecules may include another polypeptide molecule and/or a ligand molecule.
>
> The term "protein switch" is used herein to describe a multiple component system comprising (i) a first polypeptide molecule; (ii) a second polypeptide molecule which binds to the first polypeptide molecule in a manner modulatable by a ligand; and (iii) the ligand.

Thus, the ligand component of a complex comprising a heterodimer (two polypeptides) and a ligand, as described in the specification, cannot reasonably be interpreted to be a single zinc ion that does not modulate the interaction of the two polypeptides. As set forth in *In re Morris, supra,* claims must be read as they would be understood by a person of skill in the art. This hypothetical person would have understood that the ligand component of the complex of claim 34 is not a zinc ion coordinated by a zinc finger, but, rather, a molecule that is distinct from the DNA-binding polypeptides and that mediates the binding of the two polypeptide molecules to each other.

Although it is believed to be eminently clear from the specification, Appellants have no objection to claim 34 reciting that the ligand binds to both polypeptides of the claimed complex. If the examiner believes that such language is remedial, he is requested to contact the undersigned to effect such a change. Alternatively, the Board is asked to use its discretion and make a statement under 37 C.F.R. § 41.50(c) that the use of such language in claim 34 will overcome this rejection.

2. Barbas fails to describe or suggest a complex within the scope of claim 34

In rejecting claim 34 over Barbas, the Examiner has failed to take into account the enlightenment provided by the specification in regard to determining the correct meaning of the claim term "ligand." The broadest reasonable interpretation of this term, in the context of the complex of claim 34, is not a zinc ion coordinated by one of the two the DNA-binding polypeptides, but, rather a separate, molecular component that mediates interaction of the two DNA-binding polypeptides. Accordingly, the Zif$(C7)_6$-Jun/Zif-268-Fos heterodimers disclosed by Barbas are not complexes as claimed because they lack a ligand component. Barbas discloses two polypeptides (Jun and Fos), which interact directly to form a heterodimer. There is no ligand component that mediates the binding of the two polypeptides in these complexes.

B. Claim 48 would not have been obvious over the cited references

Claim 48 is finally rejected as allegedly obvious over WO 93/23431 (hereinafter "Vegeto"), as evidenced by McEwan *et al.* and Bledsoe *et al.* in light of Liu *et al.* (Final Office Action, pages 3-6). In support of the rejection, it was stated that Vegeto discloses mutated steroid hormone receptors and their uses as a molecular switch for regulating nucleic acid expression in mammals, and glucocorticoid receptors as starting material for making such mutant receptors. *Id.* It was maintained that the motivation to combine the references can be implicit and requires consideration of common knowledge and common sense. (Advisory Action, page 3, citing *Dystar Textilfarben v. C.H. Patrick*, 80 USPQ2d 1641 (Fed. Cir. 2006)). The Advisory Action continues:

...Vegeto et al. teaches a modified steroid receptor in which the native DNA binding domain is replaced and, as evidenced by McEwan et al. and Bledsoe et al., wherein dimerization is mediated by a ligand. The basis of the rejection is that **it would be obvious** to use the polydactyl DNA binding domain taught by Liu et al. in the modified steroid receptor taught by Vegeto et al. (Advisory Action, page 4, emphasis added.)

1. Claim Construction: "first" and "second" polypeptides

In support of the rejection, the examiner urges that "it would be obvious to use the polydactyl DNA binding domain taught by Liu et al. in the modified steroid receptor taught by Vegeto et al." Such a combination would result in a system which might have the capacity to form a homodimer containing two identical copies of a modified steroid receptor/zinc finger fusion protein.

In contrast, claim 48 recites a system comprising a first polypeptide and a second polypeptide. These terms must be construed to recite two different polypeptides. *See* Board Decision in Appeal No. 2006-1270 at pp.7-8 (copy attached in Related Proceedings Appendix). Thus, even if the disclosures of Vegeto and Liu were combined, they would neither disclose nor suggest the heterodimer of claim 48.

Although it is believed to be clear from the language of claim 48 and from the Board Decision in Appeal No. 2006-1270, Appellants have no objection to claim 48 reciting a heterodimer. If the examiner believes that such language is remedial, he is requested to contact the undersigned to effect such a change. Alternatively, the Board is asked to use its discretion and make a statement under 37 C.F.R. § 41.50(c) that the recitation of a heterodimer in claim 48 will overcome this rejection.

2. The rejection is improperly based on hindsight reconstruction

Appellants have never disputed that an obviousness inquiry takes into consideration common knowledge and common sense. See, Response After Final, pages 4-7, noting that the motivation to combine can derive from the common knowledge. However, an obviousness rejection is only proper if it is based on common knowledge available at the time of filing. As clearly set forth in *Dystar* and every other case regarding obviousness, the motivation to combine cannot be based on what would be obvious after the specification at issue is filed. This is improper hindsight reconstruction (*Dystar* at page 1656):

> As we recently explained in *Alza Corp. v. Mylan Labs.*, Inc., No. 06-1019, 2006 U.S. App. LEXIS 22616 [80 USPQ2d 1001] (Fed. Cir. Sept. 6, 2006), the suggestion test—as our motivation-to-combine inquiry has come to be known—"prevent[s] statutorily proscribed hindsight reasoning when determining the obviousness of an invention."

Moreover, the common knowledge relied upon must be clearly set forth by the Examiner (*Dystar* at 1649, emphasis added):

> Likewise, a close reading of *In re Lee* reveals that our objection was not to the Board's statement that "[t]he conclusion of obviousness may be made from common knowledge and common sense of a person of ordinary skill in the art without any specific hint or suggestion in a particular reference", but **its utter failure to explain the "common knowledge and common sense" on which it relied**.

In the case on appeal, the Examiner has improperly based the obviousness rejection on a finding of what would be obvious in light of Appellants' disclosure, rather than what would have been obvious at the time of filing. See, Advisory Action, reproduced above, stating that it "would be obvious" instead of the proper test of what "would have been obvious." In addition, the "common knowledge" relied upon in making this assertion has not been set forth. Rather, the rejection is founded upon an assertion that the nature of the problem to be solved by Vegeto is to regulate expression of a nucleic acid sequence in mammals, combined with Liu's disclosure of proteins theoretically capable of recognizing a unique site in a human genome.

Thus, the examiner has failed to make a *prima facie* case of obviousness, even by his own criteria. Moreover, as will be shown below, the references teach against their combination with each other and, even if they were combined, would not disclose or suggest the switching system recited in claim 48.

3. The record fails to show that dimerization of Vegeto's mutant glucocorticoid receptors is mediated by a ligand

The examiner asserts that "Vegeto et al. teaches a modified steroid receptor in which the native DNA binding domain is replaced and, as evidenced by McEwan et al. and Bledsoe et al., wherein dimerization is mediated by a ligand" (Advisory Action, page 4). However, contrary to the examiner's assertion, McEwan and Bledsoe do not show that dimerization of Vegeto's modified steroid receptor is mediated by a ligand. Rather, what McEwan, Bledsoe and the art as a whole actually teach is that, for a wild-type steroid receptor, the ligand mediates the dissociation of the receptor from a chaperone protein such as *hsp90*. For example:

> In the absence of hormone, the receptor appears to be predominantly in the cytoplasm complexed with other proteins, most notably hsp90 [*citations omitted*]. Upon binding steroid, this complex dissociates and the receptor can enter the nucleus, dimerise and bind to specific DNA sequences . . . (McEwan at page 153, first column, first paragraph of "Introduction" section)

> In the absence of ligand, GR [glucocorticoid receptor] is retained in the cytoplasm by association with chaperone proteins such as hsp90 and p23, which bind to the LBD [ligand binding domain] [*citation omitted*]. The chaperone activity of the hsp90 complex has been shown to be critical for hormone binding by GR [*citations omitted*]. Hormone binding initiates the release of chaperone proteins from GR, allowing dimerization and translocation of the receptor into the nucleus. (Bledsoe at page 93, second column, second full paragraph)
>
> Like ER, GR and its related receptors AR and PR, activate transcription as homodimers and **the GR LBD alone has been shown to be capable of forming a homodimer** [*citation omitted*]. (Bledsoe at page 94, first column, first full paragraph, emphasis added)

See also Figure 2A of Bledsoe (page 96), which shows the structure of a GR dimer, with the dimerization interface at the center of the Figure and the ligand molecules (dexamethasone) well removed from the interface and thus not mediating dimerization. Bledsoe elaborates, in the sentence bridging pages 98-99: "In the crystal structure, dexamethasone is completely enclosed within the bottom half of the GR LBD (Figure 2B)." Thus, both McEwan and Bledsoe fail to teach that dimerization of wild-type steroid receptors (let alone the mutated steroid receptor of Vegeto, see below) is mediated by their ligand, as asserted by the examiner, and consequently do not support the rejection.

4. There is no motivation to combine the references

Claim 48 is drawn to protein switches in which the dimerization of two DNA-binding polypeptides (at least one of which is an engineered Cys2-His2 zinc finger protein) is modulated by a ligand.

The references themselves fail to teach or suggest the protein switch of claim 48. First and foremost, Vegeto does not teach a protein switch as claimed that comprises two different DNA binding domains whose interaction is mediated by a ligand. Rather,

Vegeto teaches a molecular switch comprising a natural or modified steroid receptor DNA binding domain linked to a modified steroid receptor ligand binding domain (see, *e.g.*, claims 32 and 33 of Vegeto). Vegeto does not in any way teach or suggest that a ligand mediates interaction of two DNA binding domains, as required by claim 48. Furthermore, since the ligand binding domain of steroid receptors contains sequences responsible for receptor dimerization (see, page 2, lines 25-27 of Vegeto), Vegeto's modification of this domain may, in fact, destroy dimerization capability. Notably, Vegeto is silent as to whether their mutant steroid receptors form homodimers, indicating instead that interaction with the ligand sufficiently "activates" the modified receptor (Vegeto, page 9, lines 16-19):

> The term "ligand" refers to any compound which activates the receptor, usually by interaction with (binding) the ligand binding domain of the receptor. However, ligand [*sic*] can also include compounds which activate the receptor without binding.

McEwan and Bledsoe disclose dimerization of wild-type steroid receptors (see above), and therefore provide no information about the dimerization properties of Vegeto's receptors that have been mutated in the ligand binding domain. Thus, Vegeto does not teach or suggest, either explicitly or inherently, two DNA binding domains whose binding to each other is modulated by a ligand.

Liu relates to engineered zinc finger proteins -- there is nothing in this reference about ligand-mediated interaction of two such zinc finger proteins and therefore no reason to combine Liu with Vegeto as set forth by the Examiner. Indeed, while Liu relates to polydactyl zinc finger proteins for "unique addressing within complex genomes" (see, Liu, Title), all of Vegeto's disclosure relating to the use of modified steroid receptors for regulation of gene expression is directed to regulation of <u>exogenous</u> genes. Vegeto does not teach or suggest the desirability of addressing a unique site. All that Vegeto requires is the ability to recognize the transcriptional element present in the

vector in which the cassette is incorporated.[3] Accordingly, there would not have been a motivation for one of skill in the art to combine the disclosures of Vegeto and Liu.

Thus, Vegeto and Liu do not teach ligand-modulated interaction of two proteins, at least one of which is an engineered ZFP, as claimed, and there is no combination of these references that teaches or suggests systems (switches) comprising two DNA-binding polypeptide components whose interaction is modulated by a ligand, as set forth in claim 48.

Despite the failure of Vegeto and Liu to teach or suggest ligand-modulated interaction of two DNA-binding domains as claimed, the rejection was maintained on the grounds that, in the present, the art considered as a whole somehow teaches or suggests all the elements of claim 48 (Advisory Action, page 4, emphasis added):

> The basis of the rejection is that it **would be obvious** to use the polydactyl DNA binding domain taught by Liu et al. in the modified steroid receptor taught by Vegeto et al. **The rejection is based on the art considered as a whole**; therefore, it is not necessary for Liu et al. to teach the elements supplied by Vegeto et al. and *vice versa*.

However, as noted above, the proper test is not what "would be obvious" in the present, which includes the disclosure at issue. Rather, the Examiner must provide sound reasons supporting why claim 48 "would have been obvious" to the skilled artisan at the time of filing. No such evidence has been supplied. The Office has not pointed to anything in the references or the common knowledge available at the time of filing that would motivate the skilled artisan to modify Vegeto's steroid receptor-containing, single DNA-binding domain, exogenous gene-regulating system to arrive at the subject matter of claim 48, namely a system comprising two DNA-binding polypeptides in which binding of the two polypeptides to each other is modulated by a ligand. Indeed, at the time of filing, it was far from "common knowledge" that engineered zinc finger proteins could be

[3] For example, Vegeto uses the term "nucleic acid cassette" in the sections of her application related to the use of modified steroid receptors in gene regulation (*e.g.*, page 7, lines 20-27; page 17, lines 1-30). Vegeto defines "nucleic acid cassette" as genetic material that is incorporated into a cell and oriented in a vector (page 10, line 30 through page 11, line 4).

used in dimerizing protein switches as claimed and the Examiner has not provided any evidence to the contrary.

As noted above in *Dystar* and in the Response After Final, with regard to cases cited in the Final Office Action (*Sernaker* and *Fulton*), a *prima facie* case of obviousness has not been made out because the Examiner's contention that the skilled artisan would have somehow had the knowledge modify Vegeto to employ Liu's proteins is completely unsupported by any reasoning "based on established scientific principles" that some advantage would have resulted from the hypothetical modifications. It is only with Appellants' disclosure in hand that a skilled artisan would combine Vegeto and Liu. *See In re Kotzab* 55 USPQ2d 1313, 1318 (Fed. Cir. 2000) and *Amgen, Inc. v. Chugai Pharm. Co.,* 18 USPQ2d 1016, 1023 (Fed. Cir. 1991) stating that "hindsight is not a justifiable basis on which to find that the ultimate achievement of a long sought and difficult scientific goal was obvious." Accordingly, because there is no evidence that a skilled artisan would have made the suggested combination at the time of filing, the rejection is an impermissible hindsight reconstruction.

5. Vegeto teaches away from its combination with Liu

The examiner relies upon the following argument to support his motivation to combine Vegeto's mutant receptors with Liu's zinc finger proteins:

> Vegeto *et al.* does not teach that a protein should comprise a non-naturally occurring Cys2-His2 zinc finger binding domain or an engineered or mutated zinc finger binding domain. However, Vegeto et al. does teach, "In preferred embodiments of the molecular switch, the modified steroid receptor has both the ligand binding domain and DNA binding domain replaced" (p, 7, ll. 7-9; emphasis added) and suggests certain non-mammalian DNA binding domains. (Office Action dated April 18, 2006, pages 12-13.)

However, careful review of Vegeto's specification reveals that the only substitute DNA-binding domains suggested by Vegeto are non-mammalian DNA-binding domains. See Vegeto at page 7, lines 7-12 and page 16, lines 14-18. Indeed, this is admitted by the

examiner (see quote from April 18, 2006 Office Action reproduced above). Yet, while Vegeto teaches substitution of non-mammalian DNA-binding domains for the DNA-binding domain of her mutant receptors, Liu teaches design of proteins for use in mammalian cells (*i.e.*, proteins theoretically capable of binding a unique site in a human genome). Thus, the references themselves teach away from their combination with each other, and this deficiency cannot be remedied by postulating some potential advantage to be gained by combining the two references.

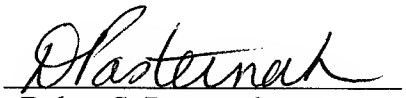
For all of the aforementioned reasons, the rejection of claim 48 under 35 U.S.C. § 103(a) should be withdrawn.

CONCLUSION

For the reasons stated above, Appellants respectfully submit that the pending claims are novel and non-obvious. Accordingly, Appellants request that the rejections of the claims on appeal be reversed, and that the application be remanded to the Examiner so that the appealed claims can proceed to allowance.

Respectfully submitted,

Date: April 23, 2007

By: 
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CLAIMS APPENDIX

The claims on appeal are as follows:

34. A complex comprising:

(a) a heterodimer comprising

(i) a first polypeptide, and

(ii) a second polypeptide; and

(b) a ligand,

wherein the first and second polypeptides bind to DNA, and further wherein the first or second polypeptide comprises an engineered Cys2-His2 zinc finger binding domain.

48. A switching system comprising a protein switch comprising: (i) a first component comprising a first polypeptide and (ii) a second component comprising a second polypeptide, in which the first polypeptide binds to the second polypeptide in a manner modulatable by a ligand, and (iii) a third component comprising the ligand, wherein the first and second polypeptides bind to DNA, and further wherein the first or second polypeptide comprises an engineered Cys2-His2 zinc finger binding domain.

EVIDENCE APPENDIX

A color copy of Bledsoe *et al.* (2002) *Cell* **110:**93-105 (cited by the examiner in an Office Action dated May 16, 2005) is attached to this Brief.

RELATED PROCEEDINGS APPENDIX

A copy of the Board's Decision in *Ex parte Cox et al.* (Appeal No. 2006-1270; USSN 10/222,614), mailed September 27, 2006 is attached. See, in particular, pages 7-8, at which construction of the terms "first protein" and "second protein" was addressed by the Board.

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Crystal Structure of the Glucocorticoid Receptor Ligand Binding Domain Reveals a Novel Mode of Receptor Dimerization and Coactivator Recognition

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Summary

Transcriptional regulation by the glucocorticoid receptor (GR) is mediated by hormone binding, receptor dimerization, and coactivator recruitment. Here, we report the crystal structure of the human GR ligand binding domain (LBD) bound to dexamethasone and a coactivator motif derived from the transcriptional intermediary factor 2. Despite structural similarity to other steroid receptors, the GR LBD adopts a surprising dimer configuration involving formation of an intermolecular β sheet. Functional studies demonstrate that the novel dimer interface is important for GR-mediated activation. The structure also reveals an additional charge clamp that determines the binding selectivity of a coactivator and a distinct ligand binding pocket that explains its selectivity for endogenous steroid hormones. These results establish a framework for understanding the roles of protein-hormone and protein-protein interactions in GR signaling pathways.

Introduction

The glucocorticoid receptor (GR) is a steroid hormone-activated transcriptional factor known to regulate, either directly or indirectly, target genes involved in glucose homeostasis, bone turnover, cell differentiation, lung maturation, and inflammation (Reichardt et al., 2000). Mutations in GR are associated with Cushing's syndrome, autoimmune diseases, and various cancers (Werner and Bronnegard, 1996). As such, GR is widely recognized as a therapeutically important target. GR ligands, including dexamethasone, prednisolone, and other related corticosteroid analogs, are commonly used to treat diverse medical conditions such as asthma, allergic rhinitis, rheumatoid arthritis, and leukemia (Barnes et al., 1998). However, clinical use of oral corticosteroids is limited by a number of side effects ranging from increased bone loss and growth retardation to suppression of the hypothalamic-pituitary-adrenal axis. Discovery of a GR agonist that retains the beneficial anti-inflammatory activities without the undesired side effects is the subject of intense pharmaceutical efforts.

GR belongs to the nuclear receptor (NR) superfamily, which includes receptors for the mineralocorticoids (MR), estrogens (ER), progestins (PR), and androgens (AR), as well as receptors for peroxisome proliferators (PPARs), vitamin D (VDR), and thyroid hormones (TR). Phylogenetic analysis and sequence alignments show that GR, MR, PR, and AR form a subfamily of oxosteroid receptors that are distinct from the ER subfamily (NRNC, 1999). Like most nuclear receptors, GR is a modular protein that is organized into three major domains: an N-terminal activation function-1 domain (AF-1), a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD). In addition to its role in ligand recognition, the LBD contains a ligand-dependent activation function (AF-2) that is tightly regulated by hormone binding.

Within the context of the full-length receptor, both the AF-1 function and the DNA binding activity of GR are dependent on hormone binding. In the absence of ligand, GR is retained in the cytoplasm by association with chaperone proteins such as hsp90 and p23, which bind to the LBD (Pratt and Toft, 1997). The chaperone activity of the hsp90 complex has been shown to be critical for hormone binding by GR (Bresnick et al., 1989; Picard et al., 1990). Hormone binding initiates the release of chaperone proteins from GR, allowing dimerization and translocation of the receptor into the nucleus. In the nucleus, GR binds to DNA promoter elements and can either activate or repress transcription depending on the context of the target promoters. In addition, GR can also crosstalk with other transcriptional factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) to repress their gene activation activities (reviewed in McKay and Cidlowski, 1999). This GR mediated repression has been postulated to be a molecular basis for the anti-inflammatory and immunosuppressive activities of glucocorticoids. Both the ligand-dependent activation and repression by GR require the intact function of the LBD.

The molecular mechanism of ligand-dependent regulation of nuclear receptors has been illustrated by crystal structures of more than a dozen NR LBDs that are either in the apo-state or bound to agonists or antagonists. (Bourguet et al., 1995; Brzozowski et al., 1997; Renaud et al., 1995; Wagner et al., 1995; Xu et al., 1999). These structures not only reveal that the LBDs fold into a canonical three-layer helical sandwich that embeds a hydrophobic pocket for ligand binding, but also highlight

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the importance of the C-terminal (AF-2) helix in ligand-dependent regulation. In the apo- or antagonist-bound receptor, the AF-2 helix is destabilized from its "active" conformation to allow the LBD to interact with corepressors such as nuclear corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT; Chen and Evans, 1995; Horlein et al., 1995). Agonist binding induces a conformational change of the AF-2 helix, stabilizing the receptor in an active conformation to facilitate its association with coactivator proteins, such as steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor 2 (TIF2; Onate et al., 1995; Voegel et al., 1996). These coactivators contain multiple LXXLL motifs, which interact with the NR LBD (Heery et al., 1997; Le Douarin et al., 1996). Various crystal structures of receptor/coactivator peptide complexes have revealed a general mode of coactivator binding to NRs. In these structures, the coactivator LXXLL motifs adopt a two-turn α helix and both helical ends are stabilized by a "charge clamp" formed in part by a conserved acidic residue from the AF-2 helix (Darimont et al., 1998; Nolte et al., 1998; Shiau et al., 1998). However, the structural basis for the sequence specific recruitment of LXXLL motifs by different LBDs has remained ambiguous.

As DNA binding transcription factors, most NRs function as dimers. Receptor dimerization is mediated in part through the LBD. Crystal structures of the ER homodimer and two different RXR heterodimers reveal a common mode of dimerization, where the N-terminal half of helix-10 from one monomer packs against the same portion of helix-10 from the other monomer in a parallel manner (Bourguet et al., 1995; Gampe et al., 2000). The dimerization is mediated through a sequence motif of $\phi\ \phi K\phi\phi\ \phi K\phi\phi$ X $\phi R\phi\phi$ (where ϕ is a hydrophobic residue and X is any residue) that forms a coiled-coil structure in the N-terminal half of helix-10. Mutations in the $\phi K\phi\phi$ repeats in ER that abolish dimerization result in a receptor that is defective for initiating of gene transcription (Valentine et al., 2000). Like ER, GR and its related receptors AR and PR, activate transcription as homodimers and the GR LBD alone has been shown to be capable of forming a homodimer (Savory et al., 2001). The dimer interface observed in the PR LBD structure does not involve helix-10 and is significantly smaller than that seen in the RXR heterodimer or in the ER homodimer (Williams and Sigler, 1998). The physiological relevance of this PR dimer interface remains to be determined. To date, the arrangement of the GR LBD dimer has not been defined. In the full-length receptor, GR contains an additional homodimer interface on the C-terminal end of the DBD. Interestingly, a mutation (A458T) in this region of mouse GR, termed GR^{dim}, is defective in transcription activation but not transrepression (Reichardt et al., 1998).

Given its biological and pharmaceutical importance, there has been enormous interest in elucidating the GR LBD structure. However, these structural efforts have been hampered by the inability to obtain a purified receptor that retains ligand binding activity. In this paper, we describe expression, purification, crystallization, and structure determination of the GR LBD in complex with dexamethasone and a coactivator motif derived from the cofactor TIF2. Surprisingly, the structure reveals a novel dimer interface unlike that observed for any other nuclear receptor. Mutagenesis studies support the importance of this dimer interface in GR function. The crystal structure also reveals an unanticipated second charge clamp that is responsible for the specificity for the third TIF2 LXXLL motif, and a distinct steroid binding pocket with features that explain ligand binding and selectivity. Since GR is highly homologous to MR, AR, and PR, the structure presented here should serve as a model for understanding the roles of ligand binding, coactivator recruitment, and receptor dimerization in the signaling pathways mediated by these steroid receptors.

Results

Purification, Characterization, and Crystallization of the GR LBD

Historically, the GR LBD has been a very difficult protein to express in a recombinant form mostly due to solubility problems. We were also unsuccessful in our own attempts to express the wild-type human GR LBD at high levels even though a wide range of conditions were explored. To overcome these difficulties, we performed a sequence alignment of GR with the related steroid receptors PR and AR, which have been previously expressed and purified from *E. coli* (Matias et al., 2000; Sack et al., 2001; Williams and Sigler, 1998). This analysis was initiated to identify residues that are hydrophobic in GR but hydrophilic in PR and AR as they might contribute to solubility or aggregation problems. We also built a structural model for GR by using its homology to the PR LBD to explore the molecular basis of the poor behavior of the GR protein and to prioritize residues for systematic mutagenesis. Strikingly, we found that a single phenylalanine to serine mutation in helix 5 (F602S) significantly improved *E. coli* expression of a soluble GR LBD (residues 521–777) in the presence of dexamethasone (Figure 1A). This point mutant LBD was purified to homogeneity for biochemical and structural studies described below.

To assess the functional activity of the purified GR LBD, we used a fluorescence polarization assay to test the binding of a fluorescently labeled dexamethasone derivative to the receptor. In this experiment, we attempted to remove the excess dexamethasone carried over from purification by extensive dialysis. Figure 1B shows that this dialyzed GR LBD exhibits dose-dependent saturable binding to the fluorescent dexamethasone derivative with an apparent affinity of 60 nM. Addition of excess unlabeled dexamethasone completely inhibited the binding signal of the labeled dexamethasone derivative.

Dexamethasone is a potent agonist that promotes the binding of coactivators to GR (Ding et al., 1998). To test the ability of the dexamethasone-bound GR LBD to recruit coactivators, we used surface plasmon resonance to measure the interaction of the receptor with a peptide containing the third LXXLL motif from TIF2 (Voegel et al., 1998). TIF2 is the human homolog of the mouse GR interacting protein 1 (GRIP1) and its third LXXLL motif has been shown to be preferred by GR (Ding et al., 1998). Figure 1C shows that addition of a 5-fold excess of dexamethasone enhances the binding

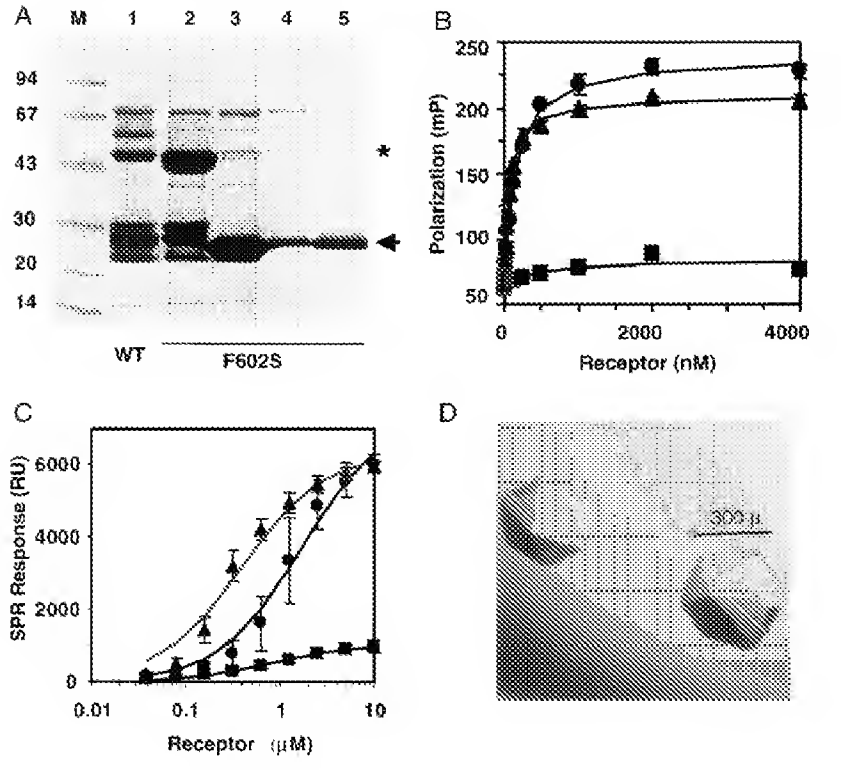

Figure 1. Purification and Characterization of the GR LBD

(A) Comparison of the protein expression of wild-type GR (lane 1) and the F602S GR (lane 2) in the presence of 10 μM dexamethasone. The proteins shown are the soluble fractions eluted from the Ni^{2+} column. Lanes 3–5 show the purification of the F602S GR LBD (lane 3, the sample after thrombin digestion; lane 4, the Ni^{2+} column flow through of the thrombin-digested sample; lane 5, the final purified protein. The molecular weight markers are shown in lane M and the 6 × HisGST-fused and cleaved GR LBD are indicated with a star and an arrow, respectively.

(B) Binding of dexamethasone to the purified GR F602S proteins as measured by fluorescence polarization assays (circles: GST-GR LBD; triangles: GR LBD; and squares: GR LBD in the presence of 100 μM unlabeled dexamethasone).

(C) Ligand-dependent binding of TIF2 coactivator motif to GST-GR LBD in the presence of a 5-fold excess of dexamethasone (triangles), RU486 (squares), and no compound (circles) was measured by surface plasmon resonance.

(D) Crystals of the GR/Dex/TIF2 complex.

of the TIF2 coactivator peptide to the purified and dialyzed GR LBD. In contrast, addition of a 5-fold excess of RU486, a known GR antagonist, inhibited the binding of the receptor to the TIF2 peptide. These results demonstrate that the purified GR LBD is able to bind either an agonist or an antagonist in the absence of the hsp90 chaperone. Importantly, the ligand-mediated association of the TIF2 peptide to the purified GR correlates with the agonist and antagonist properties of dexamethasone and RU486. Based on these results, a ternary complex of the purified GR LBD bound with dexamethasone and the TIF2 peptide was prepared and crystallized (Figure 1D).

Structure of the GR/Dexamethasone/TIF2 Complex

The GR/dexamethasone/TIF2 complex was crystallized in the P61 space group with two complexes in each asymmetric unit. Data sets were collected from two independent crystals to 2.8 Å and 2.5 Å, respectively. We determined both structures by the molecular replacement method using a GR model built from the PR LBD structure (see Experimental Procedures). The electron density map calculated with the molecular replacement solutions showed clear tracing for two GR LBD monomers (residues 523–777), two bound molecules of dexamethasone, and the LXXLL motifs of the two TIF2 peptides. The statistics of data sets and the refined structures are summarized in Table 1.

In the crystals, each GR LBD is bound to a molecule of dexamethasone and a TIF2 coactivator peptide (Figure 2A). The structure of the GR LBD contains 11 α helices and 4 small β strands that fold into a three-layer helical domain with an overall organization closely resembling the structures of PR and AR (Matias et al., 2000; Sack et al., 2001; Williams and Sigler, 1998). Helices 1 and 3 form one side of a helical sandwich whereas helices 7 and 10 form the other side. The middle layer of helices (helices 4, 5, 8, and 9) are present in the top half of the protein but are absent in the bottom half of the protein. This arrangement of helices creates a cavity in the bottom half of the GR LBD where the dexamethasone molecule is bound. The AF-2 helix, which plays an essential function in ligand-dependent activation, adopts the so-called "agonist bound" conformation where it packs against helices 3, 4, and 10 as an integrated part

Table 1. Statistics of Crystallographic Data and Structures

Crystals	1	2
X-ray Source	Rigaku-200	APS-17BM
space group	$P6_1$	$P6_1$
resolution (Å)	20.0–2.8	50.0–2.5
unique reflections	18,923	27,095
completeness (%)	99.7	99.4
I/σ (last shell)	25.7 (2.3)	35.9 (2.5)
R_{sym}[a] (%)	8.5	8.2
Refinement statistics		
R factor[b] (%)	25.4	23.7
R free (%)	30.8	26.7
rmsd bond lengths (Å)	0.015	0.007
rmsd bond angles (degrees)	1.800	1.500
total non-hydrogen atoms	4502	4845

rmsd is the root mean square deviation from ideal geometry.

[a] $R_{sym} = \Sigma \mid Iavg - Ii \mid / \Sigma Ii$

[b] $R_{factor} = \Sigma \mid F_P - F_{Pcalc} \mid / \Sigma F_P$, where F_P and F_{pcalc} are observed and calculated structure factors, R_{free} was calculated from a randomly chosen 10% of reflections excluded from refinement and R_{factor} was calculated for the remaining 90% of relfections.

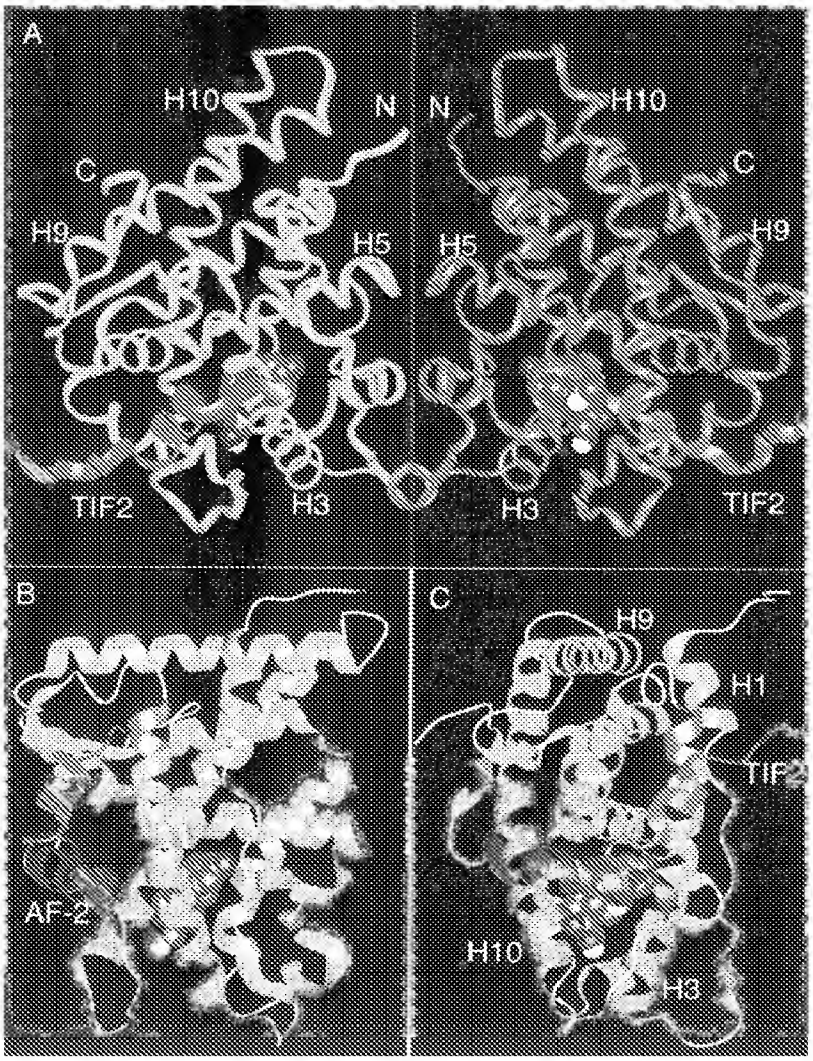

Figure 2. Structure of the GR/Dex/TIF2 Complex

(A) Overall arrangement of the GR LBD dimer. The two LBDs are shown in yellow and blue worms; the two TIF2 peptides are in purple ribbon; and the two dexamethasone molecules are in space-filling representation with carbon, oxygen, and hydrogen colored in green, red, and white, respectively. The C2 symmetry axis is shown in red.

(B and C) Two 90-degree views of the GR/Dex/TIF2 monomer complex, where helices are colored in yellow and β strands are in gold. The AF-2 helix is in red and the lysine residue from helix 3 that forms the charge clamp is in blue. The TIF2 peptides are shown in purple.

of the domain structure. Following the AF-2 helix is an extended strand that forms a conserved β sheet with a β strand between helices 8 and 9. This C-terminal β strand also appears to play an important role in receptor activation by stabilizing the AF-2 helix in the active conformation. Deletion of the last few residues that form the β strand resulted in an inactive receptor (Zhang et al., 1996).

The GR Dimer Interface

Strikingly, the two GR LBD monomers in each asymmetric unit are arranged in a unique dimer configuration. Unlike the asymmetric arrangement of the PPARγ/RXR heterodimer structures (Gampe et al., 2000), the two GR monomers show a C2 symmetric packing arrangement in which either LBD can be superimposed on the other by rotating 180 degrees around the 2-fold axis (Figure 2A). Formation of the dimer buries 623 Å^2 of solvent accessible surface in the dimer interface, which is also stabilized by a series of hydrophobic and hydrogen bond interactions (Figure 3A). The central hydrophobic interface is made up of reciprocal interactions between residues P625 and I628 in the β turn of strands 3 and 4 (Figure 3B). Surrounding this core hydrophobic interface is an extensive network of hydrogen bonds mediated by the extended strand between helices 1 and 3 (residues 547–551) and the last residue of helix 5 (Q615). In particular, residues 547–551 from each LBD, resembling two anti-parallel β strands, are in excellent geometry to form four hydrogen bonds (Figure 3C). These hydrogen bonds may also play a key role in stabilizing the GR dimer configuration.

To confirm the presence of dimeric GR LBD in solution, we analyzed the distribution of monomers and dimers in the GR population by equilibrium analytical ultracentrifugation. In the presence of both dexamethasone and the TIF2 peptide, a clear monomer-dimer equilibrium was observed with an apparent dimerization affinity (Kd) of 1.5 μM (Figure 4A). We further confirmed the presence of the GR dimer in solution by dynamic light scattering. In this experiment, the PPARγ/RXR heterodimer was used as a positive control with a measured hydrodynamic diameter of 84 Å, which is consistent with the actual size of the heterodimer observed in PPARγ/RXR crystal structure (Gampe et al., 2000). The hydrodynamic diameter of the GR LBD complex was measured to be 82 Å, which is also closely correlated with the side to side distance of 83–88 Å observed in the GR dimer structure.

Since P625 and I628 make up the core hydrophobic

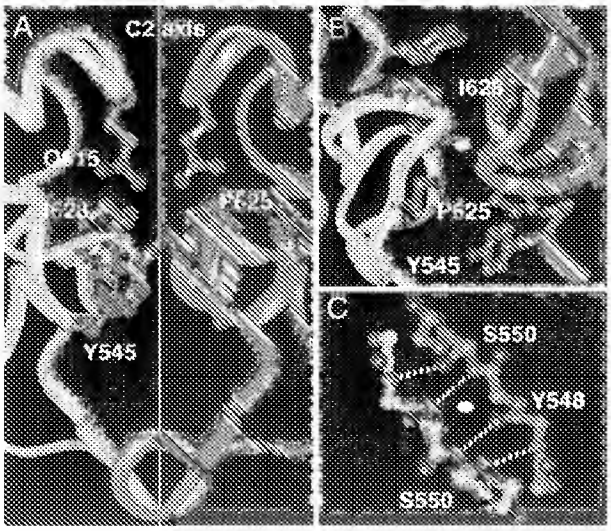

Figure 3. Characterization of the GR Dimer Interface

(A) The GR dimer interface showing the key residues and the pseudo β strands (bottom). The I628 and P625 are labeled for the two LBDs (colored in yellow and blue, respectively).

(B) The core hydrophobic interactions between the two GR LBDs. The I628 and P625 are both labeled for the blue monomer.

(C) The hydrogen bond network between the pseudo β strands.

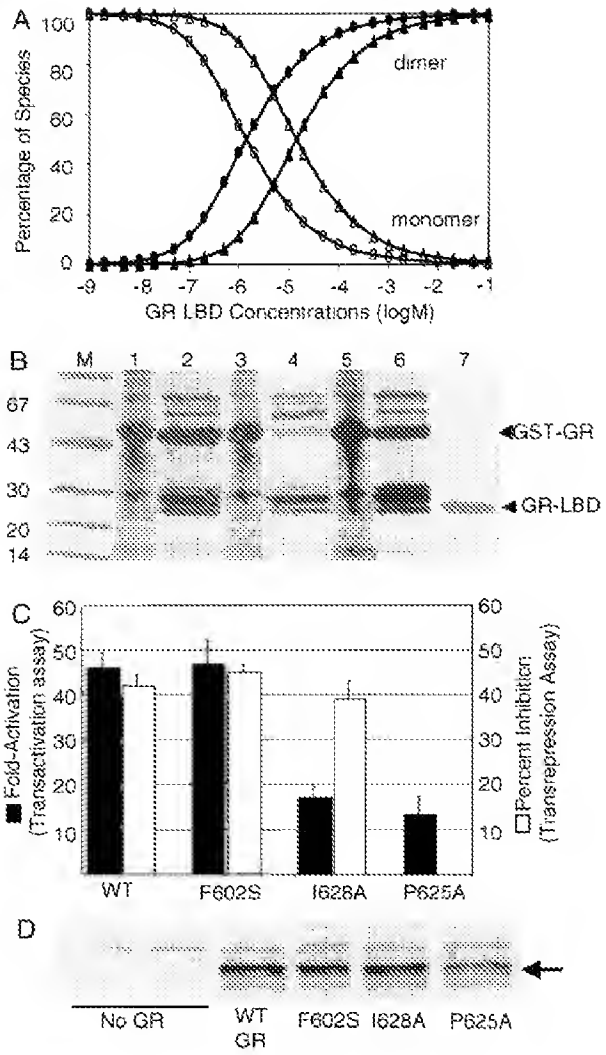

Figure 4. Functional roles of the GR Dimer Interface

(A) Dimer-monomer distributions of the F602S GR LBD (circles) and the I628A/F602S LBD (triangles) as analyzed by analytic ultra-centrifugation.

(B) Comparison of the protein solubility of the P625A/F602S mutant (lanes 3–4) and the I628A/F602S mutant (lanes 5–6) with the F602S mutant (lanes 1–2). Lanes 1, 3, and 5 represent pellet fractions and lanes 2, 4, and 6 represent soluble fractions. The purified I628A/F602S mutant LBD is shown in lane 7 with molecular weight markers indicated (lane M in Kd).

(C) Transactivation and transrepression by the wild-type and mutant GRs, where the I628A and P625A mutations are made in the dimer interface. The EC50 of dexamethasone for wild-type, F602S, I628A, and P625A receptors is 26, 28, 400, and >2000 nM, respectively. The full level of activation by the P625A mutant is only 5% of wild-type.

(D) Western blots showing the expression of GR.

dimer interface, these residues were mutated to alanine within the context of the F602S mutant to test their role in LBD-LBD dimerization. The P625A/F602S LBD protein was insoluble in *E. coli* and was not useful for biochemical studies. However, the I628A/F602S GR LBD is nearly as soluble as the F602S LBD (Figure 4B) and was purified to homogeneity for in vitro characterization (lane 7 in Figure 4B). As measured by analytical ultra-centrifugation, the I628A/F602S mutant LBD showed a 10-fold decrease in dimerization affinity as compared to the F602S GR LBD (Figure 4A). These results demonstrate that the GR LBD forms a dimer in solution and the interface observed in the crystal structure is required for effective dimerization.

Functional Analysis of GR Dimer Interface

To test the functional significance of the dimer interface observed in the GR LBD, we mutated P625 and I628 to alanine within the context of the full-length receptor. We characterized these mutants in a transient transfection assay using a reporter driven by the MMTV promoter. In the presence of dexamethasone, the wild-type and the F602S mutant receptors induced 46.0-fold and 46.8-fold activation, respectively (Figure 4C). The P625A and I628A mutant proteins were expressed at levels comparable to wild-type (Western blot in Figure 4D), but both showed a 3- to 5-fold decrease in fold of activation as compared with the wild-type. Furthermore, the absolute magnitude of activation by the P625A mutant is only 5% of wild-type. The results with the P625A mutant are consistent with a previous study where the analogous rat GR mutant (P643A) was also defective in transactivation (Caamano et al., 1998). Together, these results establish that residues comprising the dimer interface are important for the GR transactivation function.

We also tested the ability of the GR mutants to inhibit activation by NF-κB. In this assay, we used a reporter driven by the promoter from the monocyte chemoattractant protein-1 (MCP-1), which is a well-characterized NF-κB activated gene (Ping et al., 1999). In contrast to the transactivation assay, GR with the I628A mutation repressed the MCP-1 promoter activity to the same extent as the wild-type or the F602S receptor (Figure 4C). However, the P625A mutant was inactive in this assay. This defect in activity of the P625A mutant in both assays is consistent with previous studies that indicated that

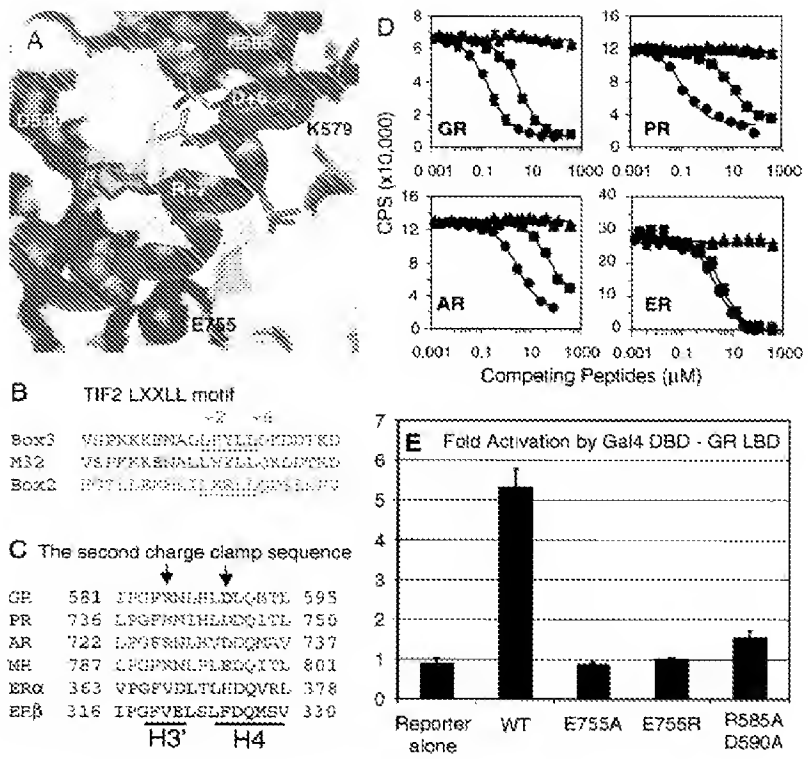

Figure 5. Structural Basis for the Specificity of Coactivator Motifs

(A) A superposition of the TIF2 third motif (purple) with the SRC-1 motif (green) on the surface of the GR coactivator binding site, where color is based on atom types (carbons: white, sulfur: yellow, nitrogen: blue and oxygen: red). The hydrogen bonds between the TIF2 residues (R+2 and D+6) and the GR residues (D590 and R585) that form the second charge clamp are indicated by green dashed lines.

(B) Sequences of the second and third coactivator motifs in TIF2, and the mutated peptide M32, where the charged residues R+2 and D+6 in the third motif have been replaced with the corresponding residues from the second motif, H+2 and Q+6.

(C) Conservation of amino acids in the second charge clamp. Sequence alignment of GR, AR, PR, and MR with arrows indicating the residues that form the second charge clamp.

(D) Effects of the R+2H and D+6Q mutation (the M32 peptide) on the binding of the coactivator motifs to GR, PR, AR, and ERβ. Dose inhibition curves are shown for the binding of the TIF2 third motif (circles) and the mutated third motif (squares) with control curves of DMSO (triangles). Compared with the wild-type TIF2 third motif, the mutated motif decreases its affinity to GR, AR, and PR by 15- to 50-fold, but has little effect (<2-fold) on binding to ERβ.

(E) Effects of the second charge clamp mutations (R585A and D590A) on the activation mediated by the GR LBD, as compared with the wild-type GR LBD or the mutations in the first charge clamp from the AF-2 helix (E755A or E755R).

the analogous rat mutation (P643A) did not translocate properly to the nucleus in the presence of ligand (Caamano et al., 1998). Importantly, the contrasting effects of I628A on transactivation versus transrepression suggest that the monomer and dimer forms of GR may regulate distinct signaling pathways.

Recognition of the TIF2 LXXLL Motif

Coactivators such as SRC-1 and TIF2 contain three LXXLL motifs, and all previous crystal structures of LBD/coactivator complexes were solved with the first or the second LXXLL motif. The GR LBD/TIF2 complex is the first structure with the third LXXLL motif, and it provides an unexpected explanation for the preferential binding of this motif to the receptor (Ding et al., 1998). In the GR LBD structure, the LLRYLL sequence in the TIF2 motif forms a two-turn α helix that orients the hydrophobic leucine side chains into a groove formed in part by the AF-2 helix and residues from helices 3, 3′, 4 , and 5 (Figure 2). The N- and C-terminal ends of the coactivator helix are clamped by E755 from the AF-2 helix and K579 in helix 3, respectively. The docking mode of the TIF2 LXXLL motif is similar to that seen in the coactivator complexes of RXR, ER, TR, PPARα, and PPARγ (Darimont et al., 1998; Gampe et al., 2000; Nolte et al., 1998; Shiau et al., 1998; Xu et al., 2001). However, unexpectedly, the GR residues D590 and R585 form a second charge clamp that interacts with residues R+2 and D+6 (Figure 5A), which are only present in the third LXXLL motif of coactivators (Figure 5B).

We addressed the functional role of the hydrogen bonds between the second GR charge clamp and the TIF2 coactivator by mutating the residues at the +2 and +6 positions of the third LXXLL motif. Replacing these two residues in the third motif with corresponding residues from the second motif decreased binding to GR (Figure 5D). Therefore, the hydrogen bonds formed with the second charge clamp contribute to the selective binding of the third TIF2 LXXLL motif. We also directly addressed the role of the second charge clamp by mutating the two charged residues D590 and R585 to alanine within the context of a GAL4-GR LBD chimeric receptor. The fusion protein of the wild-type GR LBD with the GAL4-DBD induced 5-fold activation of the reporter driven by the GAL4 DNA binding sites (Figure 5E). Mutations either in the first charge clamp (E755 on the AF-2 helix) or the second charge clamp (residues R585 and D590) dramatically reduced the activation mediated by the GR LBD, demonstrating that both charge clamps are critical for transactivation in vivo.

The residues (D590 and R585) comprising the second charge clamp in GR are conserved in PR and AR but not in ER (Figure 5C). Mutations in the third TIF2 LXXLL motif that alter the second charge clamp dramatically reduce affinity to AR and PR, similar to the result obtained with GR. These data suggest that the subfamily of oxosteroid receptors may share a common mechanism of coactivator selectivity (Figure 5D). In contrast, these TIF2 mutations have little effect on binding to ER.

Recognition of Dexamethasone

In the crystal structure, dexamethasone is completely enclosed within the bottom half of the GR LBD (Figure

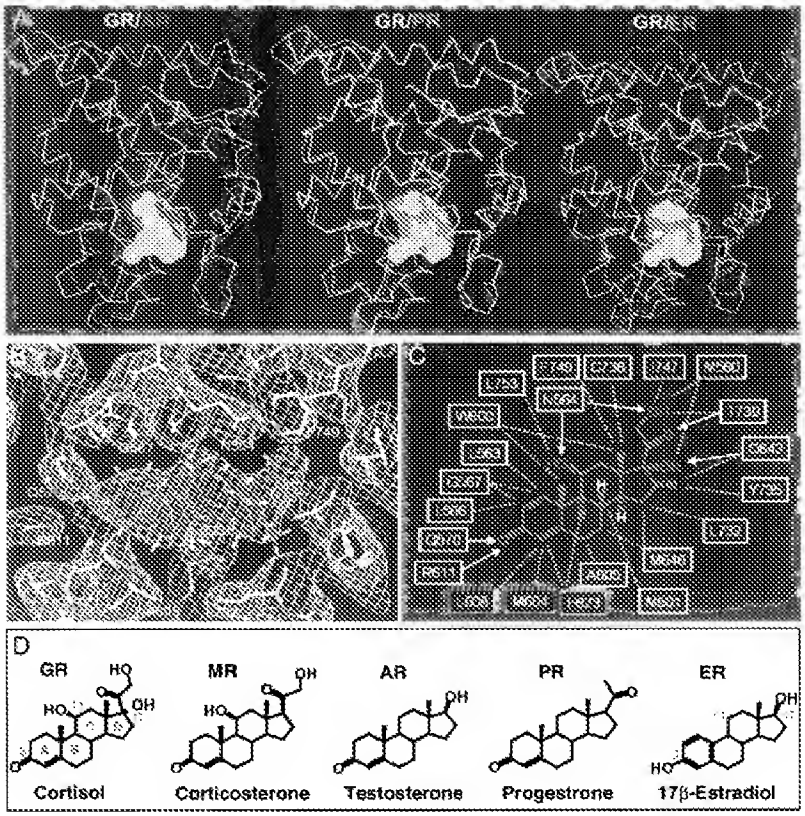

Figure 6. Molecular Basis of Ligand Recognition by GR

(A) Ligand binding pocket of GR and its comparison with the pockets of AR, PR, and ER. GR is colored in yellow in all three overlays.

(B) Electron density map showing dexamethasone and the surrounding residues in GR. The map is calculated with 2Fo-Fc coefficiency and is contoured at one σ.

(C) Schematic representation of GR/dexamethasone interactions. Hydrogen bonds and hydrophobic interactions are indicated by solid arrows and dashed lines, respectively. A possible weak hydrogen bond between C736 and the C20 ketone is omitted for clarity.

(D) Chemical structures of the endogenous steroid hormones and the numbering of the rings and key atoms.

2B). The ligand binding pocket is composed of residues from helices 3, 4, 5, 6, 7, 10, and the AF-2 helix as well as residues from the β strands 1 and 2. Strikingly, compared with the steroid-shaped pocket found in the PR, AR, or ER structures, the GR pocket has an additional branch extending from the center in the side of the steroid pocket. This additional side pocket in GR is formed by the structural rearrangement of helices 6 and 7 (Figure 6A).

The binding mode of dexamethasone can be determined unambiguously by the clear electron density (Figure 6B). The ligand is oriented with its A ring toward the β strands 1 and 2 and its D-ring toward the AF-2 helix. The volume of the GR pocket is approximately 599 $Å^3$ in subunit A and 578 $Å^3$ in subunit B. Although dexamethasone occupies only 65% of the volume, the high affinity binding of dexamethasone to GR is readily explained by the extensive hydrophobic and hydrophilic interactions between the ligand and the protein (Figure 6C). One or more hydrophobic residues within the GR protein contact nearly every atom of the steroid core of dexamethasone.

In addition, all of the hydrophilic groups of dexamethasone form hydrogen bonds with the protein. As shown in Figure 6C, the A ring carbonyl forms direct hydrogen bonds to the guanidinium of R611 and to the γ-amide of Q570. The side chain of N564 is oriented in a way to allow it to make hydrogen bonds to the C ring 11-hydroxyl and 24-hydroxyl. Furthermore, the 21-hydroxyl (off the C17 position) and the 22-carbonyl form hydrogen bonds with residues Q642 and T739, respectively. The extensive hydrogen bond network between GR and the ligand observed here are likely to contribute to the high affinity binding of dexamethasone.

Interestingly, dexamethasone also makes direct contacts with the AF-2 helix (L753) and the loop preceding the AF-2 helix (residues I747 and F749). These interactions are likely to stabilize the AF-2 helix in the active conformation, and may serve as a molecular basis for ligand-dependent activation of GR.

Discussion

A longstanding problem for GR structural studies has been the expression and purification of an active protein. Here, we have overcome this problem by using a single point mutation, F602S, in the GR LBD. This single mutation resulted in robust expression of a soluble GR LBD and ultimately allowed us to solve its crystal structure. The structure revealed a novel dimer interface, a unique steroid binding pocket, and a second charge clamp responsible for sequence specific binding of a coactivator protein. These structural observations provide critical insights into the protein-ligand and protein-protein interactions that control the GR signaling pathways.

Important Role of the GR Dimer

The GR LBD in the crystal is packed as a symmetric dimer, which is consistent with biochemical data showing that the GR LBD can form a homodimer or a heterodimer with the closely related MR LBD. These earlier studies, however, failed to identify the position or the configuration of the GR dimer interface (Savory et al., 2001). The crystallographically observed GR LBD dimer

interface is strikingly different from the helix-10 dimer interface observed in the previous ER homodimer or RXR heterodimer structures. Structure based sequence alignments reveal that RXR and its heterodimer partners have a consensus dimerization motif of ϕ AK$\phi\phi$ ϕK$\phi\phi$ X ϕR$\phi\phi$ that forms a coiled-coil structure in the first half of H10 (Gampe et al., 2000). ER and HNF4 have the same ϕX$\phi\phi$ repeats that allow them to form homodimers but lack the basic residues in the X position required for heterodimerization. However, GR and its related receptors, PR and AR, have a sequence of F YQLT KLLD S MHEV in the corresponding H10 region that is not able to form a coiled-coil structure due to the deviation of the underlined residues from the ϕX$\phi\phi$ repeats. Furthermore, in the GR structure, the extended C-terminal strand packs against the N terminus of H10 and would further block the dimer configuration seen in the ER and RXR structures (Figure 2B). The difference in the dimer interface between GR and ER may support the evolutionary divergence of ER from the oxosteroid nuclear receptors (Escriva et al., 2000).

We have also confirmed the functional significance of the GR dimer interface by mutagenesis studies. Mutations in the two interface residues P625 and I628 compromise the GR transactivation function, but show different phenotypes in transrepression. While the P625A GR is completely inactive in repression, the I628A mutant is as competent as the wild-type receptor. Using the purified I628A mutant protein, we have confirmed that this mutation decreases the LBD-LBD dimerization affinity. The phenotype of the I628A mutant is similar to that observed with the mouse GR^{dim} mutant, which shows that a defect in DBD dimerization results in loss of GR activation function without affecting transrepression activity (Reichardt et al., 1998).

The GR LBD contains multiple functions including ligand binding, chaperone association, nuclear location, transcription regulation, and others. These functions are interconnected and are highly dependent on the integrity of the three-dimensional structure of the LBD. It is possible that the phenotype of the I628A mutant can be attributed to other functions of the LBD in addition to its effect on dimerization. We have found that the I628A mutant LBD requires a 20-fold higher concentration of dexamethasone to achieve full activation as compared to the wild-type receptor (data not shown). However, the I628A mutant is still competent for nuclear localization and transrepression function at the same ligand concentration used for activation of the wild-type receptor. Thus, a mutation in the dimer interface can selectively decrease the potency for transactivation by the GR LBD.

The different phenotypes of the I628A and P625A mutants may be attributed, in part, to the behavior of their proteins. P625 is located in the β-turn between two β strands and is conserved among steroid receptors. It is likely that the P625A mutation disrupts the β-turn, thus reducing the protein stability. Although in vivo, expression of the full-length receptor either with I628A or P625A appears to be similar (Figure 4D), the P625A mutant LBD is much less soluble than the I628A mutant in *E. coli* (Figure 4B). The P625A mutant also shows defects in nuclear translocation upon ligand binding (data not shown). Our results are consistent with a previous study in which the rat GR with the corresponding mutation P643A was defective in transactivation (Caamano et al., 1998). Although the P643A rat GR retained normal ability to bind DNA, it showed decreased stability for heterocomplex formation with hsp90. Since P625 is the central dimerization residue, this would suggest that the GR dimerization interface might overlap the hsp90 binding site.

Compared with the large interface observed in the RXR dimer structures, the GR LBD dimerization interface is much more limited, reflecting its weaker dimerization affinity (Kd $\sim$1.5 μM for GR versus 1$\sim$10 nM for RXR dimers). In the context of the full-length receptor, the low dimerization affinity of the GR LBD can be compensated by an additional interface found in the DNA binding domain and possibly the hinge region preceding the LBD (Savory et al., 2001). Several reports have highlighted the role of residues in the D loop of the DNA binding domain for stabilizing receptor dimers (Luisi et al., 1991; Reichardt et al., 1998). Normal GR functions require integrity of both interfaces as a mutation in the dimer interface in the DNA binding domain also abolishes GR transactivation function (Reichardt et al., 1998). The discovery of the novel dimer interface in the LBD should provide additional ways to address the functional role of GR dimerization by genetic manipulations.

Coactivator Recognition by the Second Charge Clamp

Nuclear receptors recruit coactivators primarily through the core LXXLL motifs. The fact that there are a large number of coactivators and each contains multiple LXXLL motifs poses a question of how specific recruitment of coactivators is achieved by a given nuclear receptor. Our structure reveals that GR uses two charge clamps to define its sequence specific binding to the TIF2 third motif. The first charge clamp is composed of E755 from the AF-2 helix and K579 from helix-3, which cap the backbone amides and carbonyls of the coactivator helix. Both residues that form the first charge clamp are highly conserved across members of the NR superfamily and dictate a common binding mode for all LXXLL motifs. The second charge clamp is composed of R585 and D590, which form hydrogen bonds with the side chains of the residues R+2 and D+6 that are present in the TIF2 third motif but not in its first or second motif. These interactions with the second charge clamp are responsible, in part, for the binding specificity of GR for the TIF2 third motif. Our results are consistent with the observation that the binding specificity of this motif is encompassed within the TIF2 sequence of KENALLRYLLDKDD (Darimont et al., 1998). The residues from the second charge clamp are conserved in the oxosteroid receptors GR, AR, PR, and MR but are not present in ER or RXR-obligate heterodimers, such as PPAR. Thus, the second charge clamp may account for the differential binding of coactivator motifs by many nuclear receptors. Notably, only the second LXXLL motif is required to mediate ER transactivation while PPARγ requires both the first and the second motifs. Since proteins like SRC-1 and TIF2 function as promiscuous coactivators, the existence of three LXXLL motifs within their sequence may allow them to integrate signaling pathways across multiple

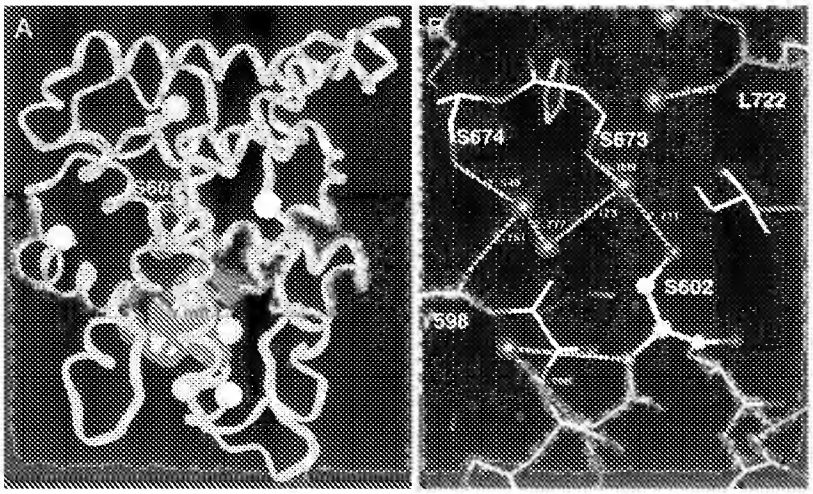

Figure 7. Basis for the GR Mutations

(A) The locations of natural GR mutations are represented as Van der Waals spheres in the overall structure. Green mutations are the residues that contact dexmethasone directly in the structure and the white mutations are in the residues that form the hydrophobic core of the protein. The side chain of S602 is also presented.

(B) Hydrogen bond network mediated by the S602 in the F602S mutant (balls and sticks) and nearby water molecules (red spheres) inside of the protein.

NRs. Alternatively, as yet undiscovered coactivators may exist that modulate GR signal transduction through selective interactions with the second charge clamp.

Hormone Selectivity by Steroid Receptors

Endogenous steroid hormones such as cortisone, testosterone, or progesterone share a similar core chemical structure (Figure 6D) but mediate distinct biological responses. Structural comparisons of GR, AR, PR, and ER have provided insight into how functional specificity is achieved by the steroid receptors. These steroid receptors share over 50% identity in their amino acid sequences and a similar three-dimensional structure. In these structures, the core steroid template (A, B, C, and D rings) assumes a common orientation with the A ring oriented toward the conserved arginine from helix-5 and the D ring toward the AF-2 helix. However, many subtle differences in the secondary structure and the topology of the ligand binding pockets exist in these steroid receptors. In particular, helices 6 and 7 of GR deviate significantly from ER, AR, and PR and produce a unique side pocket in GR (Figure 6A). This pocket may account for the GR selectivity of glucocorticoids, which have larger substituents at the C17α position compared with estrogen, progesterone, and testosterone (Figure 6D). Interestingly, the mineralocorticoids that selectively bind MR have similar substituents at the C17α position. MR may also have a similar pocket for these large C17α substitutes, since the residues that form the GR side pocket are also conserved in MR.

Besides the shape differences, the polar atoms are also distributed differently in the steroid pockets with respect to the specific protein-ligand hydrogen bonds. For example, the polar substituents in steroid hormones are mainly located at positions C3 or C17. In the GR structure, the C3 ketone accepts hydrogen bonds from Q570 and R611, which are also conserved in AR and PR. In ER, the glutamine is replaced by glutamate, which prefers to accept a hydrogen bond from the ligand, therefore accounting for ER's selectivity of a hydroxyl group at the C3 position. These differences in hydrogen bond formation may explain why GR, as well as AR, PR, and MR prefer the steroid hormones with a ketone at the C3 position, whereas ER prefers a hydroxyl group.

The selectivity of GR ligands with larger substituents at the C17 position can be best explained by the larger GR-side pocket. However, as discussed above, MR presumably has a similar pocket to GR. The selectivity of MR for mineralocorticoids can be attributed to the differences in hydrogen bonding patterns between the receptor and the ligands. In fact, the MR selective steroids, corticosterone, aldosterone, and 11-deoxylcorticosterone, all lack the 17α-hydroxyl group, which forms a specific hydrogen bond with Q642 in the GR structure. At this position, MR has a hydrophobic leucine (L848) that would disfavor the presence of a polar hydroxyl in this region. Together, the steroid selectivity appears to be achieved by the complementarity of shape and hydrogen bonding between ligands and the ligand binding pockets in the receptors

Naturally Occurring Mutations in the GR LBD

Missense mutations in the GR LBD have been associated with a number of diseases, such as Cushing's syndrome, autoimmune diseases, and various cancers, and the impact of these mutations can readily be explained by our structure. Based on the location in the GR structure (Figure 7A), these mutations can be classified into two groups. The first group includes the mutations G507C, M601L, M604P, M646T, Y735S, C736S, and L753F. In the structure, these residues are found to make direct contacts with dexamethasone (green balls, Figure 7A), and their mutations most likely result in a GR molecule that is defective in ligand binding. The second group includes mutations of P541A, I559D, C638Y, V729I, Y764N, and F774A (white balls, Figure 7A). In the structure, these residues are involved in hydrophobic interactions within the protein, and their mutations may therefore destabilize the protein. The availability of the GR structure provides an opportunity to determine whether there is any correlation in the locations of these mutations with the clinical phenotypes.

Structural Basis for the Improvement of Protein Solubility by the F602S Mutation

It is intriguing that a single point mutation of F602S has such a dramatic impact on the solubility of the GR LBD. The F602S mutation was one of fourteen mutations that

were suggested by sequence alignment and analysis of a GR homology model. Most of the mutations were targeted for lipophilic residues exposed on the surface of the protein, but had little or no effect on expression or solubility. In contrast, F602 was identified as a buried, lipophilic residue that appeared to fit poorly into its environment within GR. Remarkably, the F602S mutation substantially enhanced the soluble expression of the GR LBD. This result is consistent with the earlier work on the corresponding mutation of the rat GR (F620S), which showed increased receptor activity and was shown to be less dependent on hsp90 proteins for its function (Freeman et al., 2000; Garabedian and Yamamoto, 1992). The GR structure presented here provides a further rationale for the improvement in solubility by the F602S mutation. Although F602 is buried deeply inside of the protein at the first turn of helix-5, where it is sandwiched between helices 3 and 10 (Figure 7A), the hydrophobic side chain of F602 is surrounded by a small hydrophilic cavity (Figure 7B). This hydrophilic cavity is made up of the side chains of S599, S673, S674, and H726, and the backbone carbonyls of residues 598, 599, 670, and 722. The presence of the hydrophobic phenylalanine side chain in the polar environment would be highly unfavorable. This arrangement may cause the local instability that makes the protein prone to aggregation and/or misfolding. The F602S mutation replaces the hydrophobic phenyl ring with a small hydrophilic hydroxyl group, creating a more suitable arrangement within the hydrophilic environment, and apparently overcoming the instability problem. In the crystal structure, the volume created by the F602S mutation is filled by three water molecules. These water molecules not only cap the N terminus of helix-5 but also mediate an extensive hydrogen bond network with the residues that make up the cavity. Interestingly, PR has a serine at the corresponding position, and most of the other nuclear receptors have a glutumate residue at this position. In the structures of other NRs including PPARs, this negatively charged residue forms a pair of hydrogen bonds with a conserved arginine from the loop between helices 8 and 9 (Gampe et al., 2000; Nolte et al., 1998; Xu et al., 1999, 2001). Thus, a hydrophilic residue at position 602 has been evolutionarily conserved in other NRs, possibly to preserve the stability of the protein. GR is the only nuclear receptor with a large hydrophobic residue at this position, and this may account for the difficulty in obtaining a stable protein with wild-type GR constructs.

Extensive studies have indicated that ligand binding of GR in vivo or in vitro depends on the presence of the hsp90 chaperone complex (Pratt and Toft, 1997). It has also been proposed that hsp90 is required for opening the GR ligand binding pocket to allow ligand association (Morishima et al., 2000). Interestingly, using our purified protein, the F602S mutant GR LBD is freely accessible to agonist and antagonist as demonstrated by the competition experiment (Figures 1B and 1C). In the structure, the GR pocket is completely enclosed when it is bound with dexamethasone. Exchange of dexamethasone with RU486 in the purified protein must involve opening the pocket even in the absence of hsp90. However, the in vivo high-affinity binding of ligands still requires the chaperone activity of hsp90 (Bresnick et al., 1989; Picard et al., 1990). It is possible that the rate of ligand binding and/or exchange in vivo is dependent on hsp90. Moreover, the hsp90 complex may also function to prevent the wild-type GR protein from aggregating in vivo by maintaining the structural integrity of the otherwise unstable protein.

Experimental Procedures

Protein Preparation

The GR LBD (residues 521–777), containing a single F602S mutation, was expressed in the presence of 10 μM dexamethasone as a 6 × His-GST fusion protein from the expression vector pET24a (Novagen). The modified fusion protein contains a His-TAG (MKKGHHHHHHG) at the N terminus and a thrombin protease site between GST and the GR LBD. The GR LBD was purified to homogeneity using similar procedures previously described for AR and PR (Matias et al., 2000; Williams and Sigler, 1998). A typical yield of the purified protein is about 1 mg from each liter of cells. To prepare the protein-ligand-coactivator complex, we added a 2-fold excess of the TIF2 peptide to the purified GR LBD, which was present with 50 μM dexamethasone. The ternary complex was then diluted 10-fold with a buffer containing 500 mM ammonium acetate, 50 mM Tris, (pH 8.0), 10% glycerol, 10 mM dithiothreitol, 0.5 mM EDTA, and 0.05% β-n-octoglucoside, and concentrated to 6.3 mg/ml for crystallization. The I628A/F602 mutant LBD was expressed and purified with the same procedures for the F602S mutant LBD except the TIF2 peptide was added during purification.

Crystallization and Data Collection

The GR/dexamethasone/TIF2 crystals were grown at room temperature in hanging drops containing 3.0 μl of the above protein-ligand solutions, and 0.5 μl of well buffer containing 50mM HEPES, (pH 8.0), and 2.0 M ammonium formate. Crystals appeared overnight and continued to grow to a size up to 300 micron within a week. Before data collection, crystals were transiently mixed with the well buffer containing additional 25% of glycerol, and were then flash frozen in liquid nitrogen.

The GR/TIF2/dexamethasone crystals formed in the $P6_1$ space group, with $a = b = 126.014$ Å, $c = 86.312$ Å, $\alpha = \beta = 90°$, and $\gamma = 120°$. Each asymmetry unit contains two GR LBDs with 56% of solvent content. The 2.8 Å data set was collected with an in-house Rigaku Raxis IV detector and the 2.5 Å data set was collected with a MAR CCD detector at 17-ID in the facilities of the Industrial Macromolecular Crystallography Association (IMCA) at the Advanced Photon Source. The observed reflections were reduced, merged, and scaled with DENZO and SCALEPACK in the HKL2000 package (Otwinowski and Minor, 1997).

Structure Determination and Refinement

The 2.8 Å structure of the GR/dexamethasone/TIF2 complex was determined by molecular replacement with the AmoRe program (Navaza et al., 1992; Williams and Sigler, 1998; Xu et al., 2001). The initial GR model, containing residues 527–776 of wild-type GR and residues 740–752 of TIF2, was built with the MVP program by combining the PR LBD structure (Williams and Sigler, 1998) with the SRC-1 portion of the PPARα/SRC-1 structure (Navaza et al., 1992; Williams and Sigler, 1998; Xu et al., 2001). Two solutions were obtained from the molecular replacement search with a correlation coefficiency of 43% and an R-factor of 45.3%, consistent with two complexes within each asymmetry unit. The phases from the molecular replacement solution were extensively refined with solvent flattening, histogram matching, and 2-fold noncrystallographic symmetry (NCS) averaging as implemented in the CCP4 dm program and produced a clear map for the GR LBD, the TIF2 peptide, and the dexamethasone. Multiple cycles of manual model building, including conversion of side-chains from the SRC-1 and wild-type GR sequences to the actual TIF2 and GR F602S sequences, respectively, was carried out with QUANTA (Accelrys Inc). Structure refinements proceeded with CNX (Brunger et al., 1998), using the maximum likehood target and NCS constraints, which was relaxed in the final stages of refinement. The 2.5 Å structure of the GR/dexamethasone/TIF2 complex was then determined by using the 2.8 Å structure

as the model. The statistics of the structures and data sets are summarized in Table 1. The 2-fold symmetry axis of the GR dimer was calculated as axis of the rotation that would superimpose one LBD onto the other. The superposition was carried out using the Cα atoms from residues 530–777 of GR. Solvent accessible surface areas were calculated with the Connolly MS program and the MVP program (Connolly, 1983; Lambert, 1997). The pocket volume and binding site accessible waters were calculated with MVP.

Binding Assays

The ligand binding activity of the purified GR LBD was determined by a fluorescence polarization assay. Experiments were conducted by combining 10 nM fluorescence-dexamethasone (Molecular Probes) with increasing concentrations of the purified GR LBD in a buffer containing 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate-20 and 5 mM DTT. The fluorescence polarization values for each concentration of receptor were determined using a BMG PolarStar Galaxy fluorescence plate reader with 485 nm excitation and 520 nm emission filters. The apparent Kd values were determined with a nonlinear least squares fit of the data for a simple 1:1 interaction. Note that the apparent affinity for the binding of the fluorescent dexamethasone (60 nM) is slightly weaker than previously reported values most likely due to the presence of unlabeled dexamethasone that remains in the protein preparations despite extensive dialysis.

The binding of the TIF2 coactivator motif to the GR LBD was determined by surface plasmon resonance using a Biacore 3000 instrument. Experiments were conducted at 25°C with ~500 RU biotinylated TIF2 peptide bound onto a streptavidin chip. Running buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate-20, and 5 mM DTT) was used with a flow rate of 5 μl/min. Binding signals were determined by injecting varied concentrations of the GST-GR LBD (F602S) with no added ligand, 5-fold molar excess of dexamethasone, or RU486. A dose-response curve was constructed using the equilibrium response taken 10 s before the end of the association phase minus the response from a flow cell with no immobilized peptide.

The effects of the R+2H and D+6Q mutations in the TIF2 third motif on the binding to GR, PR, AR, and ERβ were determined by chemical mediated energy transfer assays using the AlphaScreen Technology from Packard BioScience, as described recently for nuclear receptors (Xu et al., 2002). Proteins were prepared by expressing the AR LBD (residues 662–919), the PR LBD (residues 678–933), and the GR LBD (residues 521–777 with F602S) as 6 × His-GST fusion proteins in the presence of the respective ligands R1881, progesterone, and dexamethasone. The ERβ LBD (residues 257–530) was prepared in the absence of ligand. These proteins were partially purified by Ni 2+ chromatography. The experiments were conducted with approximately 2 nM receptor LBD, 4 nM of biotinylated peptide containing the TIF2 third motif in a buffer containing 50 mM MOPS (pH 7.4), 50 mM NaF, 0.05 mM CHAPS, 0.1 mg/ml bovine serum albumin, and 5 mM dithiothreitol. An excess amount of 1 μM R1881, progesterone and dexamethasone, and estradiol was added to AR, PR, GR, and ERβ, respectively. An AlphaScreen hexahistidine detection kit was utilized and the donor and acceptor bead concentrations were 8 μg/ml. The binding signals were obtained with increasing concentrations of the unlabeled third motif peptide or the mutated peptide and detected using a Packard BioScience AlphaQuest HTS. The IC50 values were constructed from a nonlinear least squares fit of the data for a simple 1:1 interaction and are an average of four repeated experiments with DMSO as controls.

In Vitro Dimerization Assays

The solution state of the GR LBD was determined by dynamic light scattering (Protein Solution Inc) or by analytical ultra-centrifugation using a Beckman (Fullerton, CA) XL-I centrifuge. In ultra-centrifugation, 100 μl of each sample was centrifuged against 110 μl of the equivalent buffer blank using a six-sector charcoal-filled epon centerpiece in an An-60 Ti rotor. Solvent density [100 mM sodium phosphate (pH 8), and 500 mM sodium chloride] was determined empirically to be 1.035 at 4°C using a Mettler (Highstown, NJ) DA-110 density /specific gravity meter calibrated against water. The partial specific volume of the protein, $\bar{\nu}$, was calculated as described (Cohn and Edsall, 1943). Adjustments for temperature were made using the appropriate equation that has been modified to use $\bar{\nu}$ values derived for each amino acid at 25°C (Durchschlag, 1986). The partial specific volume of GR LBD was calculated to be 0.736 mL/g at 4°C. Runs were performed at 17,500, 20,000, 22,500, and 25,000 rpm at 4°C. Data sets were obtained as radial distance versus absorbance. Scans were taken at 280 nm at 1 hr intervals throughout the run. Sedimentation equilibrium was judged by the absence of change between plots of several successive scans after approximately 20–30 hr at each speed. The raw data (from the meniscus to the back of the cell) was analyzed by an adaptation of the Beckman/Microcal Origin nonlinear regression software package using multiple iterations of the Marquardt-Levenberg algorithm for parameter estimation. Multiple models were employed to determine the most accurate description of the macromolecular species.

Functional Assays of the GR Dimer Interface

Assessment of the functional activity of full-length, wild-type, and mutant GR constructs was carried out using the transient transfection assay. To assess transactivation, expression vector (pRS vector) encoding full-length, wild-type, or mutated GR protein (0.1 ng) was cotransfected into CV-1 cells with a reporter plasmid (15 ng) containing luciferase under transcriptional control of the GR-responsive region in the MMTV promoter (List et al., 1999). To assess transrepression, GR expression vectors (5 ng) were cotransfected with pCMV-4T expression vector (0.1 ng) encoding p65 NF-κB subunit, and a reporter plasmid (10 ng) containing a fragment of the NF-κB-responsive MCP-1 promoter (Ping et al., 1999). CV-1 cells were transiently transfected with lipofectamine (Invitrogen, Inc.) as described (Moore et al., 2000; Willson et al., 1996). Cells were treated with 100 nM dexamethasone on day 2 of the transfection procedure and reporter activity assessed at day 3. Luciferase activity was normalized using an internal constitutively active reporter to control for well-to-well variation. Data were plotted as fold activation by dexamethasone after subtracting background activity of CV-1 cells transfected in the absence of added receptor.

In Vivo Assays of the Second Charge Clamp Mutations

The effect of the primary and secondary charge clamp mutations on activation in vivo was determined using a Gal4 transactivation assay. The wild-type GR LBD (486–777) or mutated GR LBD was fused in frame with the Gal4 DBD in a mammalian expression vector (pSG5). The Gal4 DBD-GR LBD fusion vector (8 ng) was cotransfected into CV-1 cells with a reporter plasmid pUAS-TK (8 ng) containing luciferase under transcriptional control of the Gal4-responsive region. CV-1 cells were transiently transfected as previously described with the exception of Fugene 6 (Roche) as the transfection reagent.

Western Blotting

Equal amounts of total cellular protein were electrophoresed on 12% SDS-PAGE and transferred to Trans-Blot nitrocellulose membranes (BioRad, Hercules, CA). GR was visualized using an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NY) after incubation with rabbit polyclonal antibody (anti-human GR, sc-1002 Santa Cruz Biotechnology, Santa Cruz, CA) and a horseradish peroxidase-linked goat anti-mouse secondary antibody (Southern Biotech Associates, Birmingham, AL).

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Accession Numbers

The Protein Data Bank ID code for the structure reported here is 1M2Z.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte GEORGE NORBERT COX III, CASEY CHRISTOPHER CASE, STEPHEN P. EISENBERG, ERIC EDWARD JARVIS and SHARON KAYE SPRATT

Appeal No. 2006-1270
Application No. 10/222,614

ON BRIEF

Before GRIMES, GREEN, and LEBOVITZ, Administrative Patent Judges.

GREEN, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 114, 116 and 119-124, all of the pending claims. Claims 114 and 120-124 are representative of the claims on appeal, and read as follows:

114. A cell comprising first and second engineered zinc finger proteins, where each of the zinc finger proteins further comprises an endonuclease or functional fragment thereof, and further wherein:
(a) the first protein binds to a first target site; and
(b) the second protein binds to a second target site.

120. The cell of claim 114, wherein the cell is an animal cell.

121. The cell of claim 120, wherein the cell is a mammalian cell.

122. The cell of claim 121, wherein the cell is a human cell.

123. The cell of claim 122, wherein the cell is a stem cell.

124. The cell of claim 123, wherein the cell is a hematopoietic stem cell.

Claims 123 and 124 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. In addition, Claims 114, 116 and 119-122 stand rejected under 35 U.S.C. § 103(a) as being obvious over the combination of Brenneman[1] and Chandrasegaran.[2] After careful review of the record and consideration of the issues before us, we reverse both rejections.

DISCUSSION

Claims 123 and 124 stand rejected under 35 U.S.C. § 112, first paragraph, "as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention." Examiner's Answer, page 3.

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[1] Brenneman et al. (Brenneman), "Stimulation of intrachromosomal homologous recombination in human cells by electroporation with site-specific endonucleases," PNAS, Vol. 93, pp. 3608-12 (1996).

[2] Chandrasegaran, U.S. Pat. No. 5,792,640, issued August 11, 1998.

"[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971) (emphasis in original). "[It] is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." Id. at 224, 169 USPQ at 370. Here, the examiner has not provided "acceptable evidence or reasoning which is inconsistent" with the specification, and therefore has not met the initial burden of showing nonenablement.

The examiner engages in a Wands analysis, see In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1403 (Fed. Cir. 1988) (noting that facts that should be considered in determining whether a specification is enabling include: (1) the quantity of experimentation necessary to practice the invention, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims) to come to the conclusion that it would require

an undue amount of experimentation to make and/or use the claimed cells, wherein the cells are stem cells. See Examiner's Answer, pages 3-6.

The examiner notes that there are no working examples, and that the "specification provides guidance to use zinc finger-endonuclease fusion proteins as gene regulators . . . and as transcription repressors . . . without further elaboration as to how such functions can be achieved." Id. at 4. Those are issues that would seem to apply to the use of any cell type, not just stem cells, and the examiner does not explain how those issues provide more of an impediment to the use of stem cells as opposed to other cell types.

With respect to stem cells, the examiner focuses on the use of the zinc finger-endonuclease constructs in homologous recombination in stem cells. See id. at 4. The examiner cites Hatada[3] for the proposition "that hematopoietic stem cells have not been shown to perform homologous recombination." Id. Hanson[4] is cited for demonstrating that "hematopoietic stem cells are difficult to purify and manipulate." Id. at 4-5. Finally, Zwaka[5] is cited for the discussion that "human embryonic stem cells are more difficult to manipulate than prior art mouse embryonic stem cells," and that it does not appear that homologous

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[3] Hatada et al. (Hatada), "Gene correction in hematopoietic progenitor cells by homologous recombination," PNAS, Vol. 97, No. 25, pp. 13807-811 (2000).
[4] Hanson et al. (Hanson), "Enhanced green fluorescent protein targeted to the Sca-1 (*Ly-6A*) locus in transgenic mice results in efficient marking in hematopoeitic stem cells in vivo," Experimental Hematology, Vol. 31, pp. 159-67 (2003).
[5] Zwaka et al. (Zwaka), "Homologous recombination in human embryonic stem cells," Nature Biotechnology, Vol. 21, pp. 319-21 (2003).

recombination was known in any other human stem cell at the time of its publication. Id. at 5.

The examiner also states that

> [t]he prior art does not show human stem cells with zinc finger-endonuclease fusion proteins, the prior art therefore does not predict whether such cells could be made or used. Hatada [ ] shows that stem cells are difficult to isolate, Hanson [ ] shows that hematopoeitic stem cells are difficult to purify and manipulate, and Zwaka [ ] show[s] that human embryonic stem cells are difficult to manipulate.

Id.

As noted by appellants, see Appeal Brief,[6] page 5, the Hatada and Zwaka references relate to homologous recombination, and the claims are not limited to cells containing two engineered zinc finger proteins that also comprise an endonuclease, that are required to undergo homologous recombination. Cells comprising a single zinc finger-nuclease fusion protein, appellants assert citing Chandrasegaran, "were known to be useful in mutagenesis, targeted cleavage, gene expression, detection of conformational changes in nucleic acid and targeted recombination." Reply Brief, page 4. Thus, Hatada and Zwaka, as they relate to the frequency of homologous recombination in human stem cells, are not relevant to the issue of whether claims 123 and 124 are enabled by the specification. Moreover, while Zwaka teaches that electroporation protocols that have been developed for mouse embryonic stem cells do not achieve the same results in human embryonic stem cells, see id., abstract, the reference teaches

[6] All references to the "Appeal Brief" are to the Appeal Brief dated July 11, 2005.

further that "[f]or human embryonic stem cells, the best chemical reagents yield stable (drug-selectable) transfectants at rates about 10^{-5}," id. at page 319, first column, second full paragraph. Thus, Zwaka teaches that human embryonic stem cells may be transfected through the use of chemical reagents.

Moreover, while Hanson teaches that "[e]xperimental manipulation of hematopoietic stem cells is challenging . . . [as] [t]hey are difficult to purify, propagate ex vivo, assay, and transduce," id. at 159, second column, Hanson also teaches that enhanced green fluorescent protein was integrated into the Sca-1 (glycosyl phosphatidyl-anchored protein) locus by homologous recombination in mouse embryonic stem cells, see id., abstract. Thus, Hanson demonstrates while it may be difficult to manipulate hematopoietic stem cells, it is possible to do so. See, e.g., Johns Hopkins University v. CellPro, Inc., 152 F.3d 1342, 136-61, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998) ("The test [for undue experimentation] is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the claimed invention." (insert in original)).

Finally, the fact that the prior art does not show human stem cells with zinc finger-endonuclease fusion proteins is not the correct standard to measure enablement, for if it were, any novel and/or non-obvious invention would be, by definition, non-enabled.

Therefore, as the examiner has failed to set forth a prima facie case of unpatentability under 35 U.S.C. § 112, first paragraph, we are compelled to reverse the rejection.

Claims 114, 116 and 119-122 stand rejected under 35 U.S.C. § 103(a) as being obvious over the combination of Brenneman and Chandrasegaran.

The panel would first like to note that the rejection is premised on an incorrect claim construction.

Claim 114 is drawn to "[a] cell comprising first and second engineered zinc finger proteins, where each of the zinc finger proteins further comprises an endonuclease or functional fragment thereof, and further wherein: (a) the first protein binds to a first target site; and (b) the second protein binds to a second target site."

According to the examiner, "[t]he phrase 'A cell comprising first and second engineered zinc finger proteins' . . . is interpreted to include cells with two identical zinc finger proteins. The phrase '(a) the first protein binds to a first target site; and (b) the second protein binds to a second target site' . . . is interpreted to include two identical zinc finger target sites." Examiner's Answer, page 6.

The problem with the examiner's construction is that it is reading the limitations "first and second engineered zinc finger protein" and "a first target site" and "a second target site" out of the claims. We construe "first and second engineered zinc finger protein" as two distinct and different zinc finger proteins,

and construe "a first target site" and "a second target site" as two distinct and different target sites. Now that the claims have been construed, we now turn our attention to review of the obviousness rejection.

Brenneman is cited for disclosing "that the efficiency of homologous recombination in a human cell can be increased by digestion by an endonuclease at the site of homology." Examiner's Answer, pages 6-7. Brenneman specifically teaches that *Xba* I endonuclease, as well as the rare-cutting yeast endonuclease PI-*Sce* I increased the frequency of recombination, whereas restriction enzymes that cut outside of the repeated regions or between them "produced no change in recombination frequency." Brenneman, abstract. The examiner notes that "Brenneman [ ] does not show use of a chimeric nuclease that comprises a zinc finger protein." Examiner's Answer, page 7.

Chandrasegaran is cited for disclosing "bacterial cells transformed with a fusion protein of a three-zinc finger DNA binding domain linked to a catalytic nuclease domain of Fok I." Id. Chandrasegaran is also cited for teaching that each finger of the zinc finger protein binds to three nucleotides of a polynucleotide, and that zinc finger proteins may be designed to bind a series of triplet nucleotides of choice. See id.

The examiner concludes:

> It would have been obvious to a person of ordinary skill in the art at the time the invention was made to modify the endonuclease used by Brenneman [ ] by use of the chimeric zinc finger-Fok I endonuclease of Chandrasegaran because use of the endonuclease of Chandrasegaran allows for cleavage at other repeated sites of choice and would thereby increase the frequency

> of homologous recombination at the sites of choice. Recombination at repeated sites of choice would further enable generation of desired recombination products and allow for further study of the process of homologous recombination as exemplified by the experiments of Brenneman. Brenneman [ ] shows that cleavage at two repeated sites in a target sequence for homologous recombination increases the rate of homologous recombination, and further shows a large repeated sequence as a substrate for homologous recombination. Chandrasegaran shows that a chimeric zinc finger-Fok-I endonuclease may be designed to specifically bind to a sequence of 9 base pairs and that it is possible to design the zinc finger portion to bind to a target sequence of choice. It is further obvious from consideration of the effect of expression of endonucleases on cell viability in both Brenneman [ ] and Chandrasegaran that the lethality of the endonuclease used by both groups is due to cleavage at multiple sites in the host cell chromosome, especially in view of the observation by Brenneman [ ] that the rare-cutter PI-Sce I is not lethal to the host cell. Therefore both the cells of Brenneman [ ] and Chandrasegaran show cells with multiple target sites for endonucleolytic cleavage.

Id. at 8.

Appellants argue that "[n]owhere do the cited references, alone or in combination, teach or suggest the cells including two chimeric zinc fingers as claimed." Appeal Brief, page 9. Moreover, appellants assert that "the Examiner's interpretation that the claims 'include cells with two identical zinc finger proteins' and 'two identical zinc finger target sites' is not correct . . . [as] throughout prosecution, Appellants have repeatedly characterized the claimed subject matter as relating to 'cells comprising two zinc finger proteins of different sequence.'" Reply Brief, page 8. We agree, and the rejection must be reversed.

As we have stated above, the examiner's rejection is based on an erroneous claim construction. And as the examiner has not pointed to how the

references as combined teach or suggest a cell comprising two different engineered zinc finger proteins, wherein each protein also comprises an endonuclease, we are compelled to reverse the rejection.

CONCLUSION

Because the examiner has failed to set forth a prima facie case of unpatentability, both rejections of record are reversed.

REVERSED

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